

Enhanced expression of Fas Ligand (FasL) in the lower airways of patients with fibrotic interstitial lung diseases (ILDs)

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Abstract: The exact role of FasL, and particularly its soluble and membrane-bound forms, in the development of chronic ILDs and lung fibrosis has not been extensively explored. We aimed at analyzing membrane-bound FasL expression on alveolar macrophages (AM) and lymphocytes (AL) as well as soluble FasL (sFasL) levels in bronchoalveolar lavage (BAL) from ILDs patients, incl. pulmonary sarcoidosis (PS), hypersensitivity pneumonitis (HP), silicosis, asbestosis, idiopathic pulmonary fibrosis (IPF), nonspecific interstitial pneumonia (NSIP), and healthy subjects (n = 89, 12, 7, 8, 23, 6, 17, respectively). In IPF, significantly increased percentage of AM FasL⁺ and CD8⁺FasL⁺ cells as well as sFasL levels in BAL were found. Increased sFasL levels were also observed in HP. NSIP and asbestosis were characterized by higher AM FasL⁺ relative number; CD8⁺FasL⁺ population was expanded in asbestosis only. There was a significant decline in AL FasL⁺ percentage in PS and HP. Vital capacity was negatively correlated with sFasL levels, AM FasL⁺ and CD8⁺FasL⁺ cell relative count. CD4⁺FasL⁺ and CD8⁺FasL⁺ percentage strongly correlated with BAL neutrophilia, an unfavorable prognostic factor in lung fibrosis. The concurrent comparative BAL analysis of FasL expression indicates that FasL⁺ AM and AL (mainly Tc cells) comprise an important element of the fibrotic process, mostly in IPF. FasL might play a crucial role in other fibrosis-complicated ILDs, like NSIP and asbestosis. (*Folia Histochemica et Cytobiologica* 2011; Vol. 49, No. 4, pp. 636–645)

Key words: Fas Ligand, alveolar lymphocytes, bronchoalveolar lavage, interstitial lung diseases, lung fibrosis

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Introduction

The Fas/FasL system is considered to be the most efficient start point of the apoptosis extrinsic pathway, and therefore plays a crucial role in the regulation of cell death and survival [1]. It comprises Fas ligand

(FasL, CD95L, CD178) and its cell surface membrane receptor, Fas (FasR, CD95, APO-1). FasL is a protein existing both in a soluble (sFasL) and a cell membrane-bound form. Fas is expressed as a member of the tumor necrosis factor family of surface receptors; binding of FasL to Fas results in apoptosis of susceptible cells [2]. The process is particularly effective if FasL molecules form trimers. The Fas/FasL system is involved in the pathomechanisms of immunity and inflammation, including lymphocyte-dependent cytotoxicity, alloreactive T cells clonal deletion, virally infected or cancer cells removal as well as activation induced cell death (AICD) of T lymphocytes [3, 4]. Elevated serum sFasL concentrations have been observed in malignant and inflammatory diseases [5]. Fas is expressed by multiple cell and tissue types, whereas Fas Ligand is expressed mostly in activated T cells. It is active as one of the major effector molecules in natural killer cells and cytotoxic T lymphocytes, in particular CD8 T cells, CD4 T helper 1 (Th1) cells [6]. Consequently, in septic shock, the Fas/FasL system has been regarded as a potential therapeutic target [2].

In airways, FasL involvement was first established in the pathogenesis of acute lung injury (ALI). Matus-Bello et al. were the first to suggest in 1999 that massive release of sFasL within the respiratory system of ALI patients might trigger severe epithelial damage characteristic for ARDS [7]. Since then, the essential role of the Fas/FasL pathway in the development of lower airways apoptosis, with subsequent alveolar epithelial injury in, has been well confirmed in experimental models of lung inflammation/injury with *lpr* (i.e. Fas deficient) mice as well as in clinical studies with ALI and ARDS patients [8–11].

In contrast, the significance of FasL in chronic lower airways disorders, like interstitial lung diseases (ILDs), has not been extensively evaluated. Kuwano et al. reported an increased concentration of sFasL in BAL supernatants from patients with interstitial pneumonia associated with collagen vascular diseases (CVD-IP) and with bronchiolitis obliterans organizing pneumonia [12]. In IPF group, sFasL levels were relatively higher, but the difference compared to controls did not reach statistical significance. Nevertheless, BAL sFasL level was proposed as a useful decision marker for systemic corticosteroid therapy in IPF and CVD-IP patients [13].

Recent studies have revealed that while both forms of FasL, soluble and membrane-bound, are capable of inducing the apoptotic process, sFasL has a relatively weak pro-apoptotic effect and might actually down-regulate the activity of membrane Fas receptors [1, 14]. Thus, membrane-bound FasL expressed on cytotoxic cells seems to be more active in apopto-

sis induction than expected, due to facilitated trimerization of FasL molecules and more efficient Fas receptors ligation. In general, the role of FasL is potentially much more complex. For instance, experimental models of lung allogeneic grafts have indicated Fas ligand as a tool for immune suppression and tolerance [15].

Nevertheless, it is commonly accepted that chronic inflammatory events in lower airways, occurring in ILDs, may result in epithelial damage followed by pathological repair, fibroblast proliferation and disseminated lung fibrosis. This process is triggered by multiple proapoptotic factors, with Fas Ligand regarded as one of the most powerful [16]. The fatal ILD complication, lung fibrosis, occurs as an irreversible consequence of idiopathic pulmonary fibrosis (IPF), while in other ILD entities it is less frequent, as in NSIP or pneumoconiosis, or quite uncommon, as in Th1 lymphocyte-driven hypersensitivity pneumonitis and early pulmonary sarcoidosis [17–19].

Because of what is set out above, the present study was designed to provide insight into the Fas ligand (FasL) role in the pathomechanism of lung fibrosis in ILDs. Both Fas ligand forms, soluble as well as membrane-bound one, expressed by lower airways inflammatory cells, i.e. alveolar macrophages and lymphocytes, were analyzed in BAL material fairly representative of the local microenvironment of respiratory airways. Though the FasL functional activity was assessed indirectly, local expression was analyzed in the context of clinical data, in particular lung function tests and BAL cytoimmunological profile. We assessed reciprocal correlations between parameters characterizing FasL expression in BAL.

Material and methods

Study population. Bronchoalveolar lavage (BAL) was carried out in 145 patients with ILDs, including pulmonary sarcoidosis (PS; $n = 89$), hypersensitivity pneumonitis (HP; $n = 12$), silicosis ($n = 7$), asbestosis ($n = 8$), IPF ($n = 23$) and nonspecific interstitial pneumonia (NSIP; $n = 6$). PS and IPF patients were additionally subdivided according to their smoking status: 31 patients with PS and five with IPF were smokers. Smoking individuals with other ILDs (silicosis, asbestosis, HP and NSIP) were not enrolled into the study. PS patients with ongoing oral corticosteroid therapy ($n = 9$; prednisone 15–60 mg p.o. for at least three months), were analyzed separately. Otherwise, there was no history of previous systemic steroid or immune suppressive therapy in patients included in the study. BAL was performed with the informed consent of all patients as part of the routine diagnostics (Bioethics Committee of Nicolaus Copernicus University, approval n° KR 116/2006).

The diagnosis criteria of all examined subjects have been described previously [20]. Newly-detected PS was confirmed by typical clinical representation, a patient's specific histology (non-caseating granulomas in biopsy) and high resolution computer tomography (HRCT) findings [18]. PS patients were stratified according to conventional chest X-ray staging. HP was diagnosed on the basis of clinical data, including patient history with characteristic symptoms after allergen exposure, detection of specific antibodies in serum, typical presentation in HRCT) and results of lung function tests. In some cases, HP diagnosis was confirmed by lung biopsy. Pneumoconioses were diagnosed according to the chest X-ray standards of the International Labor Organization in subjects with professional or environmental exposure to inorganic dust, silica or asbestos, proven by anamnesis and typical findings in BAL cytology (ferruginous bodies in asbestosis and silica dust light emission in polarized optical microscopy in silicosis) [17]. The diagnosis of IPF/UIP and NSIP was based on histological features, respectively usual interstitial pneumonia, UIP, or nonspecific interstitial pneumonia, NSIP, on lung biopsy. Pathologic pertinent negative findings were considered, and other clinical conditions associated with secondary UIP or NSIP excluded. In seven patients, the diagnosis of IPF was established without a lung biopsy, according to the consensus criteria of the American Thoracic Society and European Respiratory Society [21].

The control group consisted of 17 subjects, nine non-smokers and eight smokers, diagnosed for ILD, in whom complete clinical investigation, including lung function tests, chest X-rays and HRCT finally excluded lung pathology. The control subjects did not present any signs of infection or chronic lung disease and were not on corticosteroid or any medication known as a potential ILD cause.

BAL performance. BAL cytology. BAL was performed according to European Respiratory Society guidelines, as described previously [22]. In brief, premedication with midazolam and local upper airways anesthesia with 2% lidocaine was followed by bronchofiberscopy using an Olympus Bf20. The right lung middle lobe or left lung lingula was lavaged alternatively with a volume of 200 ml NaCl 0.9% sterile solution, instilled sequentially with four 50 ml aliquots. Then, BAL fluid fractions were retrieved by gentle suction, pooled, filtered and immediately transported to the laboratory.

Fluid recovery was calculated as the percentage of instilled volume [17, 23]. The total cell count, cell viability (trypan blue exclusion test) and differential count of BAL inflammatory cells were calculated, as described before [20].

Enzyme-linked immunosorbent assay (ELISA) in BAL supernatants. Fas Ligand level measurement in BAL supernatant was performed by ELISA using a commercial kit (R&D, catalogue n° DFL00) according to the manufactur-

er's recommendations. The optical density was measured at 450 nm using spectrophotometric reader Elx800 (Biotek Instruments, Inc., USA). Fas Ligand concentration was expressed as pg/ml.

BAL immune cells phenotyping and flow cytometry (FC).

All BAL samples fulfilled the precise criteria of cytometric material acquisition and analysis [23]. Direct three-color typing was applied. In brief, BAL samples containing 50 μ l of cell suspension ($2-10 \times 10^6$ cells/ml) were incubated with saturating amounts of mouse, fluorochrome-conjugated monoclonal antibodies (MoAbs) directed against human superficial CD3, CD4, CD8, CD16, CD56, CD95 (Fas) CD178 (FasL) and CD45 antigens, (Becton Dickinson Immunocytometry Systems, Serotec) for 30 min in the dark, washed in PBS and resuspended in 300 μ l of PBS with 1% formaldehyde. Internal control consisted of samples not-stained and stained with negative isotype control (BDIS Tritest). Monoclonal set was alternatively modified for poor lymphocyte samples. FC data was acquired within 24 hours after staining, using argon ion laser 488 nm (FACSCalibur and FACScan cytometers, BDIS). The emitted light was detected through barrier filters specific for the emission range of the fluorochromes used in the study: 530/22 nm (fluorescence channel FL1) for FITC, 585/42 nm (FL2) for PE and > 650 nm (FL3) for PerCP. In each sample 8,000–12,000 cells were acquired. Alveolar macrophages (AM) and alveolar lymphocytes (AL) were gated according to cell granularity (side scatter, SSC) and intensity of CD45 staining. AL phenotype was yielded by dot plot quadrant analysis of respective fluorescence channels. AM FasL⁺ percentage was determined by window analysis of the additional sample stained with MoAbs anti-CD178 PE/CD45 PerCP. The results were presented as the percentage of gated cells. Particulars are presented in Table 1 and Figure 1 [17, 20, 23].

Statistics. Patient age and lung function test results were presented as mean \pm SD, BAL phenotype and cytology results as a median \pm SEM [23]. The Mann–Whitney U test was used to compare the data obtained in analyzed groups (untreated ILD patients vs. controls, smoker ILD subgroups vs. respective nonsmokers, corticosteroid treated PS patients vs. untreated ones). Spearman's rank correlation coefficient r_s was applied to test the correlation between two random variables; p values of less than 0.05 were considered statistically significant.

Results

The information about age, gender, lung function tests and BAL fluid recovery in the nonsmoker groups, as well as their BAL cytology data, is summarized in Table 2. Since restrictive ventilation pattern is characteristic for ILDs due to pulmonary fibrosis compli-

Table 1. Monoclonal antibodies used for BAL cell typing in flow cytometry

Sample description	FL1	FL2	FL3
Isotype control	IgG1 FITC	IgG1 PE	Anti-CD45 PerCP
	BD Pharmingen 340385 (Tritest)		
CD3/NK	Anti-CD3 FITC	(Anti-CD16 and Anti-CD56) PE	–
	BD Pharmingen 340042		
CD4/Fas/CD8	Anti-CD4 FITC BD Pharmingen 345768	Anti-CD178 PE Serotec MCA 1539	Anti-CD8 PerCP BD Pharmingen 347314
CD8/FasL/CD4	Anti-CD8 FITC BD Pharmingen 345772	Anti-CD95 PE Serotec MCA 2409	Anti-CD4 PerCP BD Pharmingen 555348
AM FasL	–	Anti-CD178 PE Serotec MCA 1539	Anti-CD45 PerCP BD Pharmingen 345809

*For materials with AL < 5% of BAL leukocytes the following samples set was applied: Tritest, anti-CD3/NK/CD45, anti-CD4(CD8)/FasL/CD45, anti-CD4(CD8)/Fas/CD45, anti-FasL/CD45 [23]

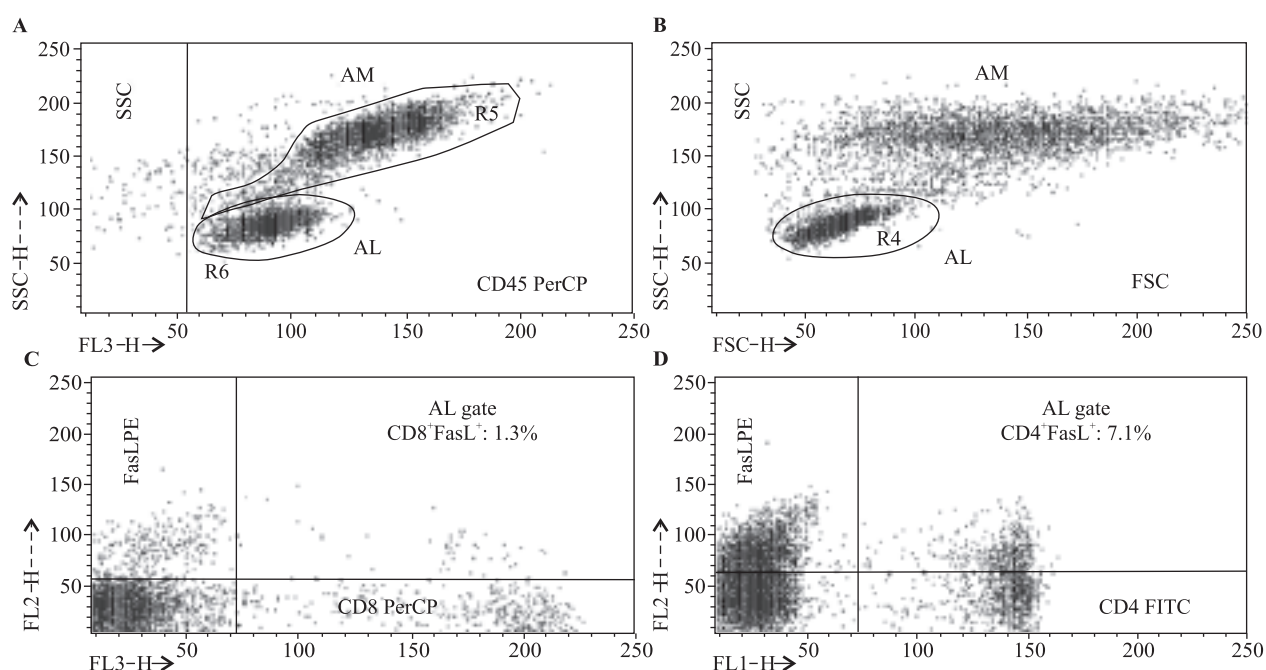


Figure 1. BAL flow cytometry. Principles of alveolar lymphocyte (AL) gating. FasL expression on AL. **A.** AL are gated as dot plot R6 according to the side scatter (SSC) and CD45 expression. Attention should be focused on AM gating possible in the same way (R5). Dots to the left of the marker are cellular debris. **B.** Back-gating. The major BAL cell populations are redefined acc. to FSC/SSC parameters (R6 dots constitute R4 gate, i.e. AL dot plot). **C.** Coexpression of CD8 and FasL on AL in PS patient: upper right quadrant dots are CD8⁺FasL⁺ cells (1.3% of AL). **D.** Coexpression of CD4 and Fas Ligand on AL in NSIP patient: 7.1% of AL. Note the high number of dots in the upper left quadrant, representing mostly CD8⁺FasL⁺ cells. All markers were set acc. to isotype negative control

cating their course, lung function analysis was limited to the vital capacity (VC) and diffuse lung capacity for carbon monoxide (DLCO) only, both presented as a percentage of predicted values (% pred). Remarkable alterations in both parameters were ob-

served in IPF and NSIP. A similar pattern, i.e. significantly impaired VC and DLCO, was present in smokers with IPF compared to smoking controls; changes in PS smokers were less pronounced. Corticosteroid-treated patients with PS (all nonsmokers, radiologi-

Table 2. Nonsmoking ILD patients and healthy controls. Selected clinical and BAL findings

Group	Male/female	Age (years)	VC (% pred)**	DLCO (% pred)**	BAL total cell no [10 ⁶ /ml]	Macrophages (AM) (%)	Lymphocytes (AL) (%)	Neutrophils (%)	Eosinophils (%)	BAL fluid recovery (%)
PS untreated	28/21	41 ± 1.3	98.3 ± 13.4	93 ± 15.3	179.5 ± 43.3*	52 ± 2.4*	44.8 ± 2.3*	0.5 ± 0.3*	0.3 ± 0.2	50.5 ± 1.7
HP	3/4	42.3 ± 2.2	103.0 ± 12.8	57 ± 16.4*	193 ± 12.3*	46 ± 8.6*	53.9 ± 6.6*	3.5 ± 3.2*	0.7 ± 0.6*	50 ± 6.6
Silicosis	7/0	56.2 ± 2.7	82.0 ± 15.9*	90 ± 11.1	370 ± 54.0*	89.2 ± 1.6	8.8 ± 1.3	1 ± 0.2	0.1 ± 0.1	47 ± 2.3
Asbestosis	7/1	57.1 ± 2.3	83.6 ± 15.2*	89 ± 15.7	375 ± 65.0*	86.5 ± 3.1	9.6 ± 2.4	2 ± 1.1*	0.3 ± 0.3	52 ± 5.4
IPF/UIP	9/7	55.8 ± 2.7	81.8 ± 14.7*	50 ± 9.0*	138 ± 44.2*	63.8 ± 5.0*	13.3 ± 3.7	9.5 ± 3.4*	2.8 ± 2.4*	48 ± 3.6
NSIP	0/6	56.2 ± 3.4	82.4 ± 12.9*	71 ± 13.7*	177 ± 34.0*	68.9 ± 7.5*	16.0 ± 5.1*	7.4 ± 6.6*	1.9 ± 1.5*	50.5 ± 2.1
Controls	5/4	45.7 ± 3.8	101 ± 12.3	105 ± 5.5	94 ± 36.7	87.3 ± 3.8	12.5 ± 2.3	0.2 ± 0.5	0.0 ± 0.2	52 ± 3.0
PS treated	2/7	51.4 ± 6.4	101 ± 17.6	80 ± 17.3*	220 ± 49.1*	59.5 ± 4.3*	38.1 ± 4.1*	3.9 ± 1.2*	0.3 ± 0.2	48 ± 4.8

*p < 0.05 in comparison with controls; **Results as percentage of predicted value; Except the last row, all involved individuals were not treated with corticosteroids

cal stages II + III) presented significantly declined VC values (data not shown).

BAL cytology results in the examined ILD groups, including the impact of tobacco consumption on BAL total cell count, cell composition, and CD4/CD8 index value, were typical for respective ILDs and consistent with previously published data [17–20, 23–25].

In nonsmokers we managed to gather sFasL results in all ILD groups (Figure 2). IPF and HP patients exhibited significantly higher sFasL levels, 2.04 ± 2.28 pg/ml ($p = 0.043$) and 13.0 ± 7.6 pg/ml ($p = 0.019$), respectively, compared to controls (0.21 ± 0.51 pg/ml). There were also relatively higher BAL sFasL concentrations in NSIP subjects, though the difference did not reach statistical significance ($p = 0.072$). Interestingly, sFasL levels observed in asbestosis were considerably increased. The limited number of analyzed patients hardly allows any interpretation, suggesting the need for further investigation.

Tobacco consumption resulted in a decline of median sFasL level in IPF smokers, compared to respective nonsmokers ($p = 0.012$). Corticosteroid therapy did not seem to affect sFasL levels in sarcoidosis patients: median values in PS stages II + III untreated and treated patients were similar.

AL immunotyping results are presented in Tables 3 and 4. Contrary to the common belief that CD8⁺ lymphocytes in the lower airways exhibit an exclusively cytotoxic profile, a relatively high proportion of BAL CD4⁺ cells expressed Fas Ligand, both in ILD patients and in controls. Moreover, while a remarkable decline in the FasL⁺ lymphocyte relative count was observed in HP and not-treated PS, CD8⁺FasL⁺ proportion was significantly higher in asbestosis and non-smoking IPF, compared to controls. It is worth noting that in the corticosteroid treated PS group, considerably higher FasL⁺ expression on AL and CD8⁺ cells in comparison with untreated PS was detected.

As a rule, the proportion of AM Fas Ligand⁺ was enhanced in most analyzed ILD groups, i.e. in non-smoking PS patients stages II + III, both steroid treated and untreated, in IPF and in NSIP (respectively, $43 \pm 5.5\%$, $51 \pm 14.8\%$, $48 \pm 8.8\%$ and $43.5 \pm 12.3\%$ vs. $23 \pm 13.6\%$ in controls). A similar tendency, though not statistically significant, was observed in PS and IPF smokers in comparison with smoking controls. The median values of FasL expression on AM were significantly higher in IPF smokers than in respective nonsmoking patients (Figure 3).

The analysis of FasL expression in the context of selected clinical and BAL cytologic data is summarized in Table 5. A significant negative correlation was observed between VC % pred and sFasL level, CD8⁺FasL⁺ cell, AM FasL⁺ as well as AL FasL⁺ rel-

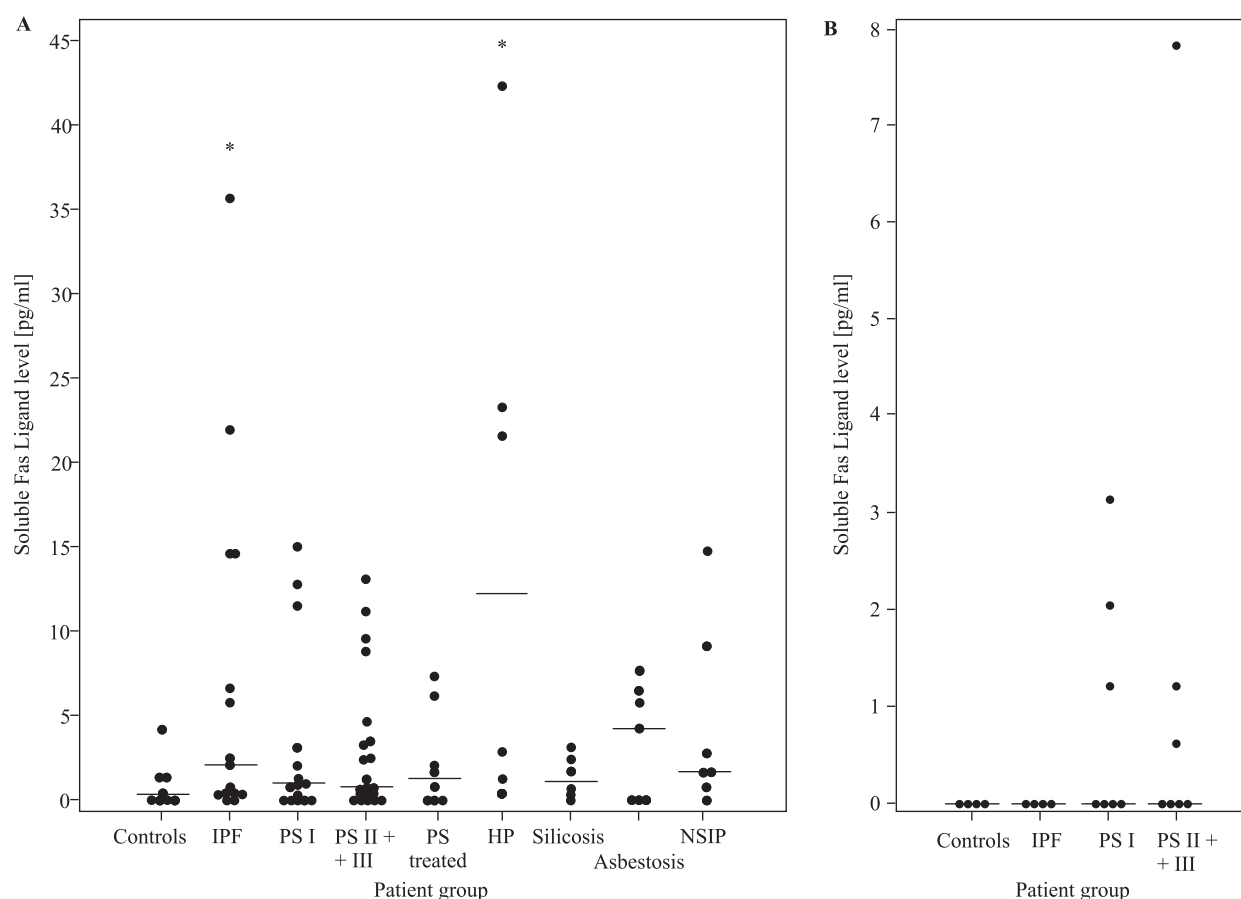


Figure 2. Soluble FasL expression in BAL supernatants. The distribution of FasL levels in nonsmoking (panel A) and smoking (panel B) subjects. Horizontal bars express median values. More particulars in the text; * $p < 0.05$ compared to controls

Table 3. Nonsmokers' FasL expression on alveolar lymphocytes (AL)

Group	n	CD4/CD8 [1]	CD4 ⁺ FasL ⁺ (%)	CD8 ⁺ FasL ⁺ (%)	BAL lymphocytes FasL ⁺ (%)
PS I	24	9.7 ± 0.8 ↑	4.8 ± 0.6	1.0 ± 0.3 ↓	6.5 ± 0.8 ↓
PS II + III	25	4.6 ± 0.9 ↑	3.8 ± 1.0	1.7 ± 0.3	7.0 ± 1.6
PS total	49	8.6 ± 0.7 ↑	4.3 ± 0.7	1.4 ± 0.3 ↓	6.9 ± 0.9 ↓
HP	7	1.1 ± 0.2 ↓	2.5 ± 2.1	2.5 ± 1.7	4.5 ± 2.7 ↓
Silicosis	7	1.8 ± 0.2	2.5 ± 0.9	4.1 ± 2.9	10.3 ± 3.3
Asbestosis	8	2.9 ± 0.7	9.3 ± 3.7	8.3 ± 1.3 ↑	21.0 ± 5.8 ↑
IPF/UIP	18	1.2 ± 0.4 ↓	4.5 ± 1.2	3.7 ± 0.8 ↑	12.8 ± 1.5
NSIP	5	1.1 ± 0.5	7.3 ± 3.2	3.2 ± 0.8	13.7 ± 2.7
Controls	9	2.1 ± 0.5	5.3 ± 2.0	2.0 ± 0.8	9.6 ± 2.3
PS treated (II + III)	9	2.7 ± 1.6 ▼	5.4 ± 1.0	5.8 ± 1.1	15 ± 2.4 ▲

Results presented as median ± SEM; ↑ $p < 0.05$ increased; ↓ $p < 0.05$ decreased compared to controls; ▲ $p < 0.05$ increased; ▼ $p < 0.05$; decreased compared to untreated PS II + III

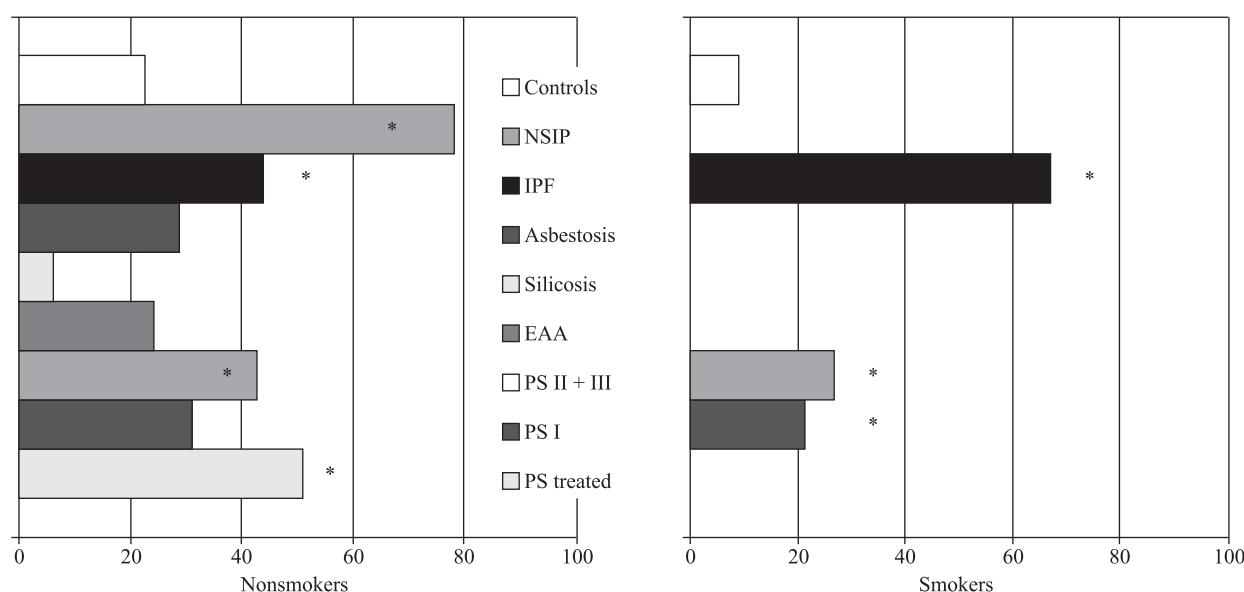
ative counts. DLCO % pred correlated significantly with soluble FasL concentration only. AL relative count was positively strongly related to soluble FasL levels and AM FasL⁺ percentage, while negatively to

CD8⁺FasL⁺ cells. BAL neutrophil relative count significantly positively correlated with FasL expression on both alveolar CD4⁺ and CD8⁺ cells, but not with soluble FasL levels.

Table 4. Smokers' FasL expression on alveolar lymphocytes (AL)

Group	n	CD4/CD8 [1]	CD4 ⁺ FasL ⁺ (%)	CD8 ⁺ FasL ⁺ (%)	BAL lymphocytes FasL ⁺ (%)
PS I	17	7.5 ± 1.0 ↑	4.7 ± 0.7	1.6 ± 0.7 ↓	8.2 ± 1.3 ↓
PS II + III	14	3.1 ± 0.3 ↑	4.9 ± 0.6	2.0 ± 0.7 ↓	8.5 ± 2.1 ↓
PS total	31	4.3 ± 0.6 ↑	4.8 ± 0.5	2.0 ± 0.5 ↓	8.0 ± 1.1 ↓
IPF/UIP	5	0.9 ± 0.2	6.4 ± 1.6	8.2 ± 2.6	18.1 ± 3.3
Controls	8	0.9 ± 0.1	3.4 ± 1.3	7.3 ± 1.7	15.0 ± 2.6

Results presented as median ± SEM; ↑ p < 0.05 increased; ↓ p < 0.05 decreased compared to controls

**Figure 3.** FasL expression on alveolar macrophages (AM). Percentage of positive AM. Results presented as medians of resp. group; *p < 0.05 in comparison with controls**Table 5.** Fas Ligand expression in lower airways versus selected clinical parameters and BAL cytology

Parameter		VC % pred	DLCO % pred	AM % of BAL cells	AL % of BAL cells	Neutrophils % of BAL cells
AM FasL ⁺ (%)	P	0.02	NS	0.01	0.05	NS
	Rs	-0.23		-0.20	+0.15	
AL FasL ⁺ (%)	P	0.01	NS	NS	0.02	0.0005
	Rs	-0.23			-0.17	+0.25
Th (CD4 ⁺) FasL ⁺ (%)	P	NS	NS	NS	NS	0.01
	Rs					+0.19
Tc (CD8 ⁺) FasL ⁺ (%)	P	0.003	NS	0.001	0.002	0.002
	Rs	-0.19		+0.24	-0.27	+0.22
Soluble FasL [pg/ml]	P	0.008	0.03	0.000002	0.000003	0.06
	Rs	-0.27	-0.30	-0.40	+0.38	+0.16

P — probability; Rs — Rank Spearman; AL — alveolar lymphocytes; AM — alveolar macrophages; Correlation results are presented for p < 0.10, significant correlations (p < 0.05) are marked in bold

In Table 5 we do not show data demonstrating excellent positive reciprocal correlations between AM FasL⁺, AL CD4⁺FasL⁺ and AL CD8⁺FasL⁺

(p < 0.0000001 for all). This strongly suggests common powerful mechanisms underlying FasL expression on immune cells in ILD lower airways.

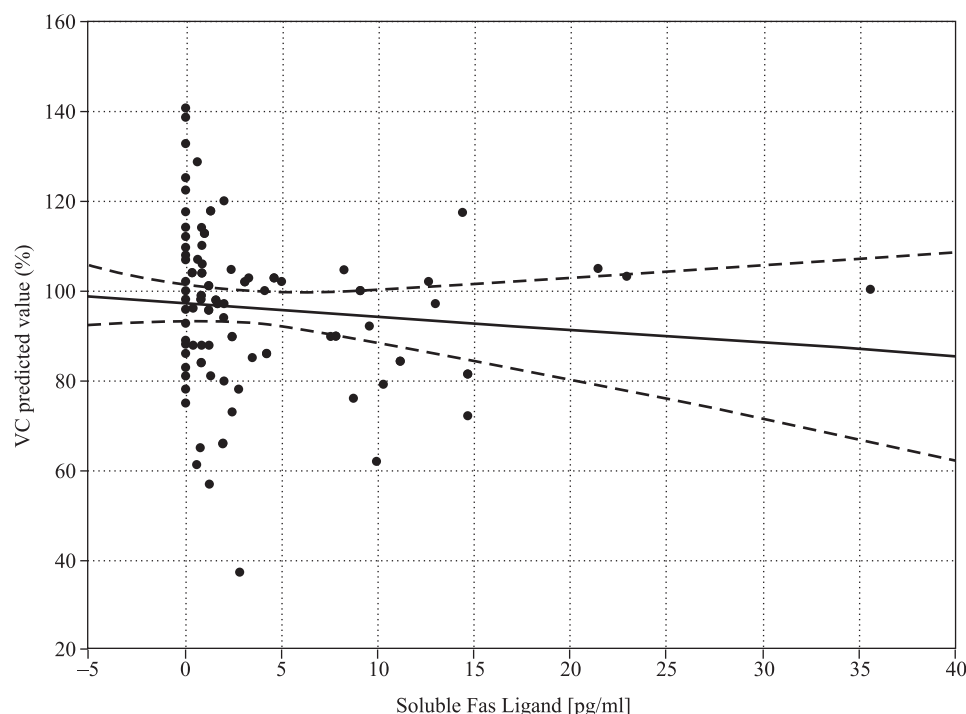


Figure 4. Correlation between sFasL expression in BAL supernatant and VC predicted value; $p = 0.008$; $RS = -0.27$

We do not present particular results of AL typing for NK cell marker ($CD3-CD16^{+}56^{+}$), CD3 and CD95 expression. Close to 100% of AL expressed CD95 without any significant differences. Similarly, both in ILDs and controls, ~90% of AL were $CD3^{+}$ with only a few NK cells [17, 23]. AL T and NK cell count did not correlate with lung function tests or FasL expression parameters.

Discussion

The close relationship between FasL and lung injury in acute lower airway disorders was proven long ago [9]. Some evidence has suggested also a direct link between pulmonary fibrosis and Fas Ligand expression in the lower airways. However, previously published studies concentrated mostly on IPF and collagen vascular diseases with concurrent ILD and examined levels of soluble FasL only. Surprisingly, patients' smoking status and ongoing therapy were not taken into account. Moreover, the examined groups were too small to provide credible data for statistical analysis. Still, Kuwano et al. were able to demonstrate that IPF and CVD patients referred for prednisolone therapy presented significantly higher sFasL levels in BAL supernatants, compared to those with a better prognosis and no indications to therapy [13]. Although the CVD group was not homogeneous and definite recommendations for systemic corticosteroid therapy in IPF have not been yet estab-

lished, the data presented by Kuwano et al. is of considerable importance.

Nonetheless, to the best of our knowledge, the current study is the first to provide simultaneous analysis of the membrane-bound and the soluble forms of FasL in the BAL material from the wide range of interstitial lung diseases with consideration to systemic corticosteroid treatment and the smoking status of the examined population.

The key finding presented here is the significantly higher concentration of sFasL observed in BAL supernatants of IPF and HP patients, with a tendency towards it in NSIP. We are the first to report this phenomenon in HP and NSIP groups, while data concerning IPF patients is in line with previous reports [13]. Not surprisingly, sFasL was not increased in sarcoidosis, which is infrequently complicated by lung fibrosis.

In our study, we suggest considerable FasL involvement in the pathogenesis of asbestosis. But as mentioned previously, a much larger group of patients needs to be evaluated in order to provide credible data.

In our opinion, the significant negative correlation between sFasL levels in BAL supernatants and lung function tests characterizing restriction pattern of ventilation disorders (VC % pred and DLCO % pred), as well as the relationship between VC % pred and membrane expression of FasL on AM and Tc cells demonstrated in the study, are of considerable inter-

est. But these results should be reevaluated in the context of experimental data suggesting the complex role of FasL in inflammatory processes. Thus, soluble FasL levels might reflect the activation status of immune cells rather than the local pro-apoptotic capacity [3]. As mentioned above, the membrane-bound FasL form is potentially more efficient in triggering apoptosis of target cells than the soluble one. Such conclusions are supported by our results collected in smokers with IPF and corticosteroid treated PS patients. In the IPF smoker group, sFasL levels were comparable to the results detected in nonsmoking patients. Furthermore, while in many BAL samples of IPF smoking patients, sFasL concentration was undetectable, it is well known that there is a high risk of extremely unfavorable disease outcome in smokers [19]. In corticosteroid treated PS, contrary to our expectations, soluble FasL levels were not lower than in untreated counterparts.

Thus, the results presented in our study seem to disagree, at least to a certain extent, with the theoretical model of FasL pathogenetic role in IPF and other ILDs. Still, hypothetically, FasL⁺ immune cells, including alveolar T cells, in particular T cytotoxic cells and alveolar macrophages, might comprise the most powerful pro-apoptotic and/or cytotoxic component of the FasL system expressed in lower airways. To the best of our knowledge, our study is the first to provide compelling analysis of FasL expression in AM population. Interestingly, experimental data seems to substantiate considerable AM pro-apoptotic activity against respiratory epithelia, higher than in other inflammatory cells [26]. According to our results, AM FasL⁺ subpopulation was significantly increased in IPF (in particular in smokers, an IPF subgroup with an extremely poor prognosis), NSIP and stages II–III both treated and untreated PS patients.

In our study ILD entities characterized by Th1 prevalence and lymphocytic alveolitis, as HP and untreated PS, presented decreased FasL expression on alveolar Th and Tc cells. A strong correlation between sFasL levels and AL relative count was also observed. Therefore, we believe that the relationship between sFasL expression and BAL lymphocyte accumulation reported in some studies might reflect FasL shedding from the AL membrane rather than the direct impact of FasL on lymphocyte recruitment [1, 9]. Additionally, in our data, the highest proportion of AL FasL⁺ was shown in IPF (for CD8) and asbestosis (for both CD8 and total AL). Also, PS patients on corticosteroid therapy (usually due to threatening lung fibrosis) presented higher AL FasL expression compared to untreated PS. The VC % pred values correlated negatively with both AM FasL⁺ and Tc FasL⁺.

Consequently, we suggest that FasL⁺ expression on alveolar lymphocytes and macrophages might reflect pro-apoptotic potential of local immune reaction, decisive for subsequent progression to lung fibrosis.

This concept is nicely supported by the strong correlation between FasL expression on AL, both CD4⁺ and CD8⁺, and BAL neutrophil relative count demonstrated in our study. It is consistent with some previous reports of neutrophil recruitment by Fas Ligand to the inflammation site [27]. It has been shown as well that membrane-bound FasL could induce neutrophil infiltration in mice. Thus, FasL chemotactic activity towards human and murine neutrophils has been implicated, though the detailed mechanism remains uncertain [2]. Consequently, the correlation between BAL neutrophil number and FasL expression is an intriguing finding, as BAL neutrophilia, in contrast to lymphocytosis, is an important unfavorable prognostic factor in ILDs [17, 19, 28]. Our observations certainly require further investigation. However, they are important due to the possible practical applications [29]. Quite recently, Chung et al. proposed the inhibition of the Fas/FasL system as a successful treatment against septic shock. The rationale of such an approach was to block the excess of lymphocyte loss, and to limit neutrophil accumulation [30].

In conclusion, it is worth emphasizing that our study provides compelling data demonstrating the upregulation of all FasL system components in IPF. However, we believe that the pathogenic role of FasL in other fibrosis-complicated ILDs, such as NSIP, asbestosis and advanced pulmonary sarcoidosis, is very likely as well. Most importantly, we provide data showing that tobacco consumption negatively affects sFasL soluble levels, but does not diminish the rate of sFasL⁺ inflammatory cells in BAL. Finally, the presented results accentuate the potential role of FasL⁺ alveolar lymphocytes and macrophages in Fas-dependent triggering of epithelial apoptosis and lung fibrosis, as well as suggesting that neutrophils and FasL⁺ cytotoxic lymphocytes may act as triggers of lung fibrosis in ILDs.

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References

1. Chávez-Galán L, Arenas-Del Angel MC et al. Cell death mechanisms induced by cytotoxic lymphocytes. *Cell Mol Immunol.* 2009;6:15–25.

2. Kavurma MM, Khachigian LM. Signaling and transcriptional control of Fas ligand gene expression. *Cell Death Differ.* 2003;10:36–44.
3. Ramaswamy M, Cleland SY, Cruz AC, Siegel RM. Many checkpoints on the road to cell death: regulation of Fas-FasL interactions and Fas signaling in peripheral immune responses. *Results Probl Cell Differ.* 2009;49:17–47.
4. Strasser A, Jost PJ, Nagata S. The many roles of FAS receptor signaling in the immune system. *Immunity.* 2009;30:180–192.
5. Tanaka M, Suda T, Haze K et al. Fas ligand in human serum. *Nature Med.* 1996;2:319–322.
6. Suda T, Okazaki T, Naito Y et al. Expression of the Fas ligand in cells of T cell lineage. *J Immunol.* 1995;154:3806–3813.
7. Matute-Bello G, Liles CW, Steinberg KP et al. Soluble Fas ligand induces epithelial cell apoptosis in humans with acute lung injury (ARDS). *J Immunol.* 1999;163:2217–2225.
8. Hashimoto S, Kobayashi A, Kooguchi K et al. Upregulation of two death pathways of perforin/granzyme and FasL/Fas in septic acute respiratory distress syndrome. *Am J Respir Crit Care Med.* 2000;161:237–243.
9. Matute-Bello G, Winn RK, Jonas M et al. Fas (CD95) induces alveolar epithelial cell apoptosis in vivo. Implications for acute pulmonary inflammation. *Am J Pathol.* 2001;158:153–161.
10. Albertine KH, Soulier MF, Wang Z et al. Fas and Fas ligand are upregulated in pulmonary edema fluid and lung tissue of patients with acute lung injury and the acute respiratory distress syndrome. *Am J Pathol.* 2002;161:1783–1796.
11. Chopra M, Reuben JS, Sharma AC. Acute lung injury: apoptosis and signaling mechanisms. *Exp Biol Med.* 2009;234:361–371.
12. Kuwano K, Miyazaki H, Hagimoto N et al. The involvement of Fas-Fas ligand pathway in fibrosing lung diseases. *Am J Respir Cell Mol Biol.* 1999;20:53–60.
13. Kuwano K, Kawasami M, Maeyama T et al. Soluble form of Fas and Fas ligand in BAL fluid from patients with pulmonary fibrosis and bronchiolitis obliterans organizing pneumonia. *Chest.* 2000;118:451–458.
14. Wesche-Soldato DE, Swan RZ, Chung CS, Ayala A. The apoptotic pathway as a therapeutic target in sepsis. *Curr Drug Targets.* 2007;8:493–500.
15. Bohana-Kashtan O, Civin CI. Fas ligand as a tool for immunosuppression and generation of immune tolerance. *Stem Cells.* 2004;22:908–924.
16. Uhal BD. Apoptosis in lung fibrosis and repair. *Chest.* 2002;122:293–298.
17. Chłap Z, Czarnobilska E, Kosiński P et al. *Cytoimmunological atlas of bronchoalveolar lavage (BAL)*. Ed. Medycyna Praktyczna, Krakow 2000.
18. *Sarcoidosis*. Ed. by Drent M, Costabel U. ERS Monographs 2005; Vol. 10, 32.
19. Selman M, Thannickia VJ, Prado A. Idiopathic pulmonary fibrosis: pathogenesis and therapeutic approaches. *Drugs.* 2004;64:405–430.
20. Kosiński P, Śladek K, Szczeklik J et al. Expression of insulin-like growth factor-I (IGF-I) in the lower airways immune cells. Evaluation of its possible role as an antiapoptotic agent. *Folia Histochem Cytobiol.* 2006;44:249–258.
21. American Thoracic Society/European Respiratory Society International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias. This joint statement of the American Thoracic Society (ATS), and the European Respiratory Society (ERS) was adopted by the ATS board of directors, June 2001 and by the ERS Executive Committee, June 2001: *Am J Respir Crit Care Med.* 2002;165:277–304.
22. Klech H, Pohl W. Technical recommendations and guidelines for bronchoalveolar lavage (BAL). *Eur Resp J.* 1989;2:561–585.
23. Kosiński P, Szczeklik J, Lackowska B et al. Flow cytometric characteristics of alveolar lymphocytes obtained by bronchoalveolar lavage (BAL) in the control group — proposal of normal value range of AL subsets in nonsmokers. *Central Eur J Immunol.* 2004;29:63–72.
24. Domagała-Kulawik J, Hoser G, Kosiński P et al. Flow cytometric evaluation of lymphocyte subpopulations in BALF of cigarette smoking healthy persons. *Folia Histochem Cytobiol.* 1999;37:25–30.
25. Kosiński P, Szczeklik J, Balicka-Ślusarczyk B et al. Modifications of the cytoimmunological pattern of bronchoalveolar lavage (BAL) material caused by cigarette smoking in selected lower air-way diseases [In Polish]. *Przegl Lek.* 2010; 67:866–870.
26. Seitz DH, Perl M, Mangold S et al. Pulmonary contusion induces alveolar type 2 epithelial cell apoptosis: role of alveolar macrophages and neutrophils. *Shock.* 2008;30:537–44.
27. Dupont PJ, Warrens AN. Fas ligand exerts its pro-inflammatory effects via neutrophil recruitment but not activation. *Immunology.* 2006;120:133–139.
28. Chorostowska-Wynimko J, Leśniewska-Radomska D, Krychniak-Soska A et al. Cellular components of bronchoalveolar lavage correlate with lung function impairment and extra-pulmonary involvement markers in active sarcoidosis. *J Physiol Pharmac.* 2004;55,3:41–47.
29. Wortinger MA, Foley JW, Larocque P et al. Fas ligand-induced murine pulmonary inflammation is reduced by a stable decoy receptor 3 analogue. *Immunology.* 2003;110:225–233.
30. Chung CS, Song GY, Lomas J, Simms HH et al. Inhibition of Fas/Fas ligand signaling improves septic survival: differential effects on macrophage apoptotic and functional capacity. *J Leukoc Biol.* 2003;74:344–51.

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